

Pharmacological Effects of Gemfibrozil on Some Isolated Smooth Muscle Preparations Of Experimental Animals

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ABSTRACT

Background: Gemfibrozil is a member of fibrates (gemfibrozil, fenofibrate, cefprofibrate, and benzafibrate) which is employed for treatment of dyslipidemia particularly hypertriglyceridemiae through its action on peroxisome proliferator activated receptors (PPAR- α). **Objective:** The aim of this work was to study the site of action and pharmacological effects of different doses of gemfibrozil on some isolated smooth muscles preparations of experimental animals. **Materials and Methods:** The experiments were conducted on isolated jejunum of rabbits, isolated spiral tracheal and urinary bladder strips of guinea pigs. **Results:** I- On isolated rabbit jejunum, gemfibrozil produced a dose-dependent reduction on the amplitude of jejunal contractions. The inhibitory effect of gemfibrozil was not abolished after complete blockade of alpha and beta adrenergic receptors, while it was completely abolished after inhibition of nitric oxide synthase by N-methyl L-arginine. On the other hand the stimulatory effects of nicotine small dose, acetylcholine, calcium gluconate, histamine and serotonin were not abolished after administration of gemfibrozil. II- On isolated tracheal spiral strips of guinea pigs, gemfibrozil produced a dose-dependent relaxation on the basal tone and a dose-dependent reduction on the amplitude of acetylcholine-induced tracheal contractions of the tracheal strips. The inhibitory effect of gemfibrozil was completely abolished after inhibition of nitric oxide synthase by N-methyl L-arginine. Gemfibrozil completely abolished also serotonin-induced contraction, while it has no effect on histamine or calcium-induced tracheal contractions. III- On isolated urinary bladder strips of guinea pigs, gemfibrozil produced a dose-dependent reduction on the amplitude of urinary bladder contractions. The inhibitory effect of gemfibrozil was not abolished after complete blockade of beta adrenergic receptors, while it was completely abolished after inhibition of nitric oxide synthase by N-methyl L-arginine. On the other hand the stimulatory effects of acetylcholine and serotonin were not abolished after administration of gemfibrozil. **Conclusion:** Gemfibrozil (antidyslipidemic, PPAR- α agonist) reduced jejunal and urinary bladder contractions and has a relaxant effect on tracheal basal tone. So it has a beneficial effect in obstructive airway diseases and cases of urgency and frequency of micturation and urinary incontinence. However, it may be used cautiously in cases of GIT disturbances as constipation and prostatic hypertrophy.

Key words: Gemfibrozil, isolated smooth muscle, jejunum of rabbits, tracheal strip, and urinary bladder strips of guinea pigs.

INTRODUCTION

Gemfibrozil (member of fibrates) is PPAR- α agonist which improves lipid profiles particularly very low density lipoprotein and high density lipoprotein in patients with dyslipidemia¹.

Peroxisome proliferator activated receptors (PPARs) are steroid receptors that exhibit broad tissue expression pattern^{3,4}. They regulate the expression of a wide array of genes that are involved in fatty acids uptake and oxidation, inflammations and vascular function⁵. The intensive studies of PPARs have revealed their importance in both normal physiology and pathology of various tissues⁶. *Phelps and Peuler* reported that fibrates has

direct smooth muscle relaxant effect through inhibition of duodenal and urinary bladder contractions⁷. This is in agreement with the study of *Nijkamp et al.* reported that gemfibrozil has a relaxant effect on the tracheal basal tone. Generally, the mechanism of the inhibitory effect of gemfibrozil on different smooth muscles is still unclear. So this work aims to study the effect of gemfibrozil on some isolated smooth muscles of jejunum, trachea and urinary bladder, and its possible mechanism of action⁸.

MATERIAL AND METHODS

Gemfibrozil was supplied in the form of tablets which were film coated. Each tablet

contained 600mg gemfibrozil which was dissolved in 60ml distilled water⁹. ***It was tested on the following strips:***

I- Experiments on isolated rabbit jejunum¹⁰:

The effect of test drug on jejunal contractions was obtained using gradually increasing doses of gemfibrozil (25-800 µg/ml). Effect of gemfibrozil (100 µg/ml) on the amplitude of jejunal contractions was studied before and after complete blockade of either α -adrenoceptors or β -adrenoceptors. Also, effects of gemfibrozil (100 µg/ml) on the stimulant effect of nicotine (1 µg/ml), acetylcholine (0.05 µg/ml), calcium gluconate (300 µg/ml), histamine (0.05 µg/ml) and serotonin (0.1 µg/ml) on the amplitude of jejunal contractions was studied. Further the inhibitory effect of gemfibrozil (100 µg/ml) on the jejunal contractions were studied before and after inhibition of nitric oxide synthase by N-methyl L-arginine (100 µg/ml):

II-Experiments on isolated tracheal spiral strips of guinea pigs¹¹ :

The effect of gemfibrozil (40-1280 µg/ml) on the tracheal basal tone against acetylcholine (3 µg/ml) induced contractions was tested. Effect of gemfibrozil (320 µg/ml) on either serotonergic receptors, histaminic receptors or calcium channels were conducted. The inhibitory effects of gemfibrozil (320 µg/ml) on acetylcholine (3 µg/ml) induced contractions of isolated tracheal spiral strips of guinea pigs were studied before and after inhibition of nitric oxide synthase by N-methyl L-arginine (100 µg/ml):

III-Experiments on isolated urinary bladder strips of guinea pigs¹² :

The effect of gemfibrozil (40-1280 µg/ml) on the amplitude of urinary bladder contractions was tested. The Effect of gemfibrozil (320 µg/ml) on the amplitude of urinary bladder contractions was recorded before and after blocking β -adrenergic receptors. The effect of gemfibrozil (320 µg/ml) on muscarinic receptors and serotonergic receptors was studied. Also, the inhibitory effect of gemfibrozil (320 µg/ml) on the amplitude of urinary bladder contractions was studied before and after inhibition of nitric oxide synthase by N-methyl L-arginine (100 µg/ml).

Statistical analysis: Statistical analysis was done using SPSS 14.0 for windows. Significant value was considered when p value <0.05. Student t-test of significant was used¹³.

RESULTS

I- On isolated rabbit jejunum:

Effect of gemfibrozil (25-800 µg/ml) on the amplitude of jejunal contractions (cm): Gemfibrozil (25-800µg/ml) produced a dose-dependent reduction on the amplitude of jejunal contractions (Fig. 1). The mean percent reduction \pm SEM ranged from 13.91 \pm 1.97 to 81.97 \pm 2.52 was found to be statistically significant while the mean percent reduction \pm SEM (3.63 \pm 1.50) was found to be statistically insignificant (Table 1). Gemfibrozil (100 µg/ml) produced reduction in the amplitude of jejunal contractions after complete blockade of alpha- and beta-adrenergic receptors (Fig. 2, 3). The stimulant effect of either nicotine small dose (1 µg/ml), acetylcholine (0.05 µg/ml), calcium gluconate (300 µg/ml), histamine (0.05 µg/ml) or serotonin (0.1 µg/ml), on the amplitude of jejunal contractions was not abolished after administration of gemfibrozil (100 µg/ml) (Fig. 4,5,6,7,8). The inhibitory effect of gemfibrozil (100 µg/ml) on the amplitude of jejunal contractions was completely abolished after inhibition of nitric oxide synthase by N-methyl L- arginine (100 µg/ml) (Fig. 9).

II- On isolated tracheal spiral strips of guinea pigs:

-Effect of gemfibrozil on the basal tone of tracheal spiral strips of guinea pigs:

Gemfibrozil (40-1280 µg/ml) produced relaxant effect on the basal tone of tracheal spiral strips of guinea pigs in a dose-dependent manner (Fig. 10). The mean relaxant effect \pm SEM ranged from 0.06 \pm 0.02 to 0.16 \pm 0.01 was found to be statistically significant, while the mean relaxant effect \pm SEM (0.03 \pm 0.02) was found to be statistically insignificant (Table 2). On acetylcholine (3 µg/ml) induced contractions (cm), gemfibrozil at small doses (40 and 80 µg/ml) did not produce any effect, while at doses (160- 1280 µg/ml) produced a dose-dependent reduction on the amplitude of acetylcholine induced tracheal contractions (Fig. 11). The mean percent reductions \pm SEM ranged from 15.54 \pm 2.53 to

55.44±3.61 which were found to be statistically significant (Table 3). Also, Gemfibrozil (320 µg/ml) completely abolished the serotonin (5 µg/ml) induced contractions while it has no effect on histamine (3 µg/ml) or calcium gluconate (50 µg/ml) induced contractions of isolated tracheal spiral strips of guinea pigs (Fig. 12,13,14). The inhibitory effect of gemfibrozil (320 µg/ml) on acetylcholine (3 µg/ml) induced contraction of isolated tracheal spiral strips of guinea pigs which was completely abolished after inhibition of nitric oxide synthase by N-methyl L-arginine (Fig. 15).

III- On isolated urinary bladder strips of guinea pigs:

-Effect of Gemfibrozil (40-1280 µg/ml) on amplitude of urinary bladder contractions (cm): Gemfibrozil (40-1280 µg/ml) produced a dose-dependent reduction on the amplitude of urinary bladder contractions (Fig. 16). The mean percent reductions±SEM ranged from 7.29±2.44 to 98.54±1.04 which were statistically significant (Table 4). Gemfibrozil (320 µg/ml) produced reduction on the amplitude of urinary bladder contractions after complete blockade of beta adrenergic receptors. The stimulant effect of either acetylcholine (3 µg/ml) or serotonin (0.1 µg/ml) on the amplitude of urinary bladder contractions was not abolished after administration of gemfibrozil (320 µg/ml). (Fig. 17,18,19) The inhibitory effect of gemfibrozil (320 µg/ml) on the amplitude of urinary bladder contractions was completely abolished after inhibition of nitric oxide synthase by N-methyl L-arginine (100 µg/ml) (Fig. 20).

DISCUSSION

In this work the experiments on isolated rabbit jejunum revealed that gemfibrozil (25-800 µg/ml) produced reduction on the amplitude of jejunal contractions in a dose-dependent manner. This result was in agreement with that of *Peuler and Phelps* who reported that gemfibrozil immediately inhibited the contraction of duodenum of rabbit¹⁴. In addition, *Phelps and Peuler* mentioned also that the amplitude of duodenal spontaneous contractions was inhibited by gemfibrozil⁷.

The mechanism of this inhibitory effect which was studied in the present work suggested that it was through stimulation of NO synthase. This mechanism may explain what reported by

Phelps and Peuler who concluded that gemfibrozil has direct smooth muscle relaxant properties¹⁵. In addition, *Phelps and Peuler* reported that gemfibrozil can directly inhibit the force of spontaneously occurring phasic rhythmic smooth muscle contractions and its primary site of action is the smooth muscle itself, either by inhibiting its responsiveness to endogenous contractile substances and/or its inherent ability to contract spontaneously on its own⁷.

The effect of gemfibrozil on isolated jejunum of rabbits represented reasonable explanation for common gastrointestinal side effects manifested by gemfibrozil treated patients; abdominal pain, dyspepsia, nausea, vomiting and constipation^{16, 2}.

In the present work the experiments on isolated tracheal spiral strips of guinea pigs showed that gemfibrozil (80-1280 µg/ml) revealed significant relaxation on the basal tone and also, gemfibrozil (160-1280 µg/ml) revealed significant reduction on the amplitude of acetylcholine induced contractions. The inhibitory effect of gemfibrozil (320 µg/ml) on acetylcholine induced contractions was completely abolished after inhibition of NOS and completely inhibited serotonin-induced contractions.

The relaxant effect on the basal tone by gemfibrozil was supported by study of *Becker et al.* which had revealed that perfusion through the lumen of guinea pig tracheal tubes in vitro with NOS inhibitors resulted in a significant increase in basal tone, suggesting a role for NO in the maintenance of basal tone¹⁷.

The inhibitory effect of gemfibrozil on acetylcholine induced contractions which was completely abolished after inhibition of NOS in this work is in agreement with the study of *Becker et al.* who reported that fenofibrate (PPAR-α agonist) decreased mice airway reactivity to methacholine and mice treated with fenofibrate and administered L-nitro arginine methyl ester¹⁷. L-NAME; (NOS inhibitor) exhibited similar reactivity to methacholine as in non treated mice suggesting that NO mediate fenofibrate induced decrease in airway reactivity. The same authors measured the level of eNOS in the lung from mice treated with fenofibrate and found that its level remains unchanged, but its activation through phosphorelation severely increases¹⁷

The nitric oxide in the airway is released from epithelial cells, nerve, and inflammatory cells and modulate the responsiveness of airway smooth muscle to acetylcholine^(18,19). The mechanism by which NO relaxes the airway is shown by *Janssen et al.* who reported that NO induced broncho relaxation via direct activation of Ca^{2+} activated K^+ channels (KCa) through covalent interaction with the cytoplasmic side of their alpha subunit⁽²⁰⁾. *Janssen et al.* added that NO relaxes the airway through cGMP dependant Ca^{2+} independent pathway²⁰.

The inhibitory effect of gemfibrozil on serotonin induced tracheal contractions in the present work was explained by the study of *Perez-Zoghbil et al.* who has reported that airway that were contracted in response to serotonin has been relaxed in response to the NO donor²¹. The same authors reported also that NO induces airway smooth muscle cell (SMC) relaxation via activation of soluble guanylate cyclase (GC) to increase cGMP that, in turn, activates protein kinase G (PKG). Activation of PKG results in the inhibition of inositol triphosphate (IP_3 R) and Ca^{2+} release from the sarcoplasmic reticulum (SR). This inhibition resulted in a decrease in the frequency of agonist-induced Ca^{2+} oscillations. The decrease in the frequency of Ca^{2+} oscillations is predominately responsible for airway relaxation²¹. Moreover, *Schlossmann et al. and Ammendola et al.* referred to the molecular mechanism by which NO/cGMP/PKG lead to inhibition of IP_3 receptors. They explained this through IP_3 receptor -associated cGMP kinase substrate protein called IRAG which has been identified in tracheal smooth muscle cells. They stated that this protein is phosphorylated by PKG and blocks IP_3 R activation by IP_3 and Ca^{2+} ^{22,23}.

In this work, the experiments on isolated urinary bladder strips of guinea pigs showed that gemfibrozil (40-1280 $\mu\text{g}/\text{ml}$) revealed significant reduction in the amplitude of urinary bladder contractions. The observed relaxant effect of gemfibrozil in the present work was in agreement with *Phelps and Peuler* who reported the inhibitory effect of gemfibrozil on the amplitude of spontaneous bladder contractions begins immediately and lasts for 30 minute⁷. In addition, the study of the same authors provided the first evidence

that fibrate drug can directly inhibit the force of spontaneously occurring phasic rhythmic smooth muscle contractions.

From another point of view, *Hove et al.* reported that NO induced relaxation in mouse aorta via two pathways, i.e. non cGMP and cGMP dependent. The first one involved a non cGMP-dependent stimulation of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), causing Ca^{2+} reuptake into the sarcoplasmic reticulum and was prominent when intracellular Ca^{2+} was mobilized²⁴. This pathway was supported by *Liu et al.* who mentioned that after incubation of vascular smooth muscle cells (VSMCs) in calcium free solution⁹. They found no difference in the level of calcium when exposed to gemfibrozil, but the intracellular calcium level decreases, suggesting that the disturbance of intracellular calcium by gemfibrozil is not related to calcium influx. The same authors reported the other pathway involves soluble guanylate cyclase and formation of cGMP, causing relaxation without changing $[\text{Ca}^{2+}]_i$, but desensitizing the contractile apparatus. This pathway related to L-type Ca^{2+} influx, and L-type Ca^{2+} channel blockers which increase the vasodilator efficacy of NO⁹. *Adachi* supported the previous mechanism by reporting that SERCA which regulates intracellular Ca^{2+} levels by pumping Ca^{2+} into stores, is a major cGMP independent target for NO⁽²⁵⁾. *Plane* reported that relaxation induced by NO is closely correlated with repolarization of the smooth muscle membrane potential²⁶. *Suzuki et al.* added also that NO donors inhibit Na^+, K^+ -ATPase activity²⁷.

A variety of mechanisms have been proposed by which NO participates in the regulation of smooth muscle free $[\text{Ca}^{2+}]_i$, which is the primary determinant of contractile tone²⁵. It is either NO increases Ca^{2+} removal from cytoplasm by accelerating the plasma membrane Ca^{2+} ATPase, or NO reduces $[\text{Ca}^{2+}]_i$ by inhibiting the agonist induced release of Ca^{2+} from intracellular stores, or the stimulatory effect of NO on $\text{Na}^+/\text{Ca}^{2+}$ exchanger, or NO induced membrane hyperpolarization by direct or indirect cGMP dependant activation of Ca^{2+} dependent K^+ channels, or the inhibitory effect of NO on L-type Ca^{2+} channels by cGMP dependant mechanism^{26,27}. NO may activate K^+ channels directly, or produced initial rapid decrease in

[Ca²⁺]_i in smooth muscle which is accounted for by the uptake of Ca²⁺ by sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) into intracellular stores which proposed that the refilling of the stores inhibits store-operated Ca²⁺ influx through non-L-type Ca²⁺ conducting ion channels and this maintains the decrease in [Ca²⁺]_i and NO induced relaxation²⁷.

Finally the NO mediated mechanism of gemfibrozil in this study is supported by different studies of many authors. *Omae et al.* reported that fenofibrate induced endothelium dependent vasodilation mediated by NO release²⁸. In addition, *Khazaei et al.* reported also that benzafibrate (pan-PPAR agonist) significantly increases serum nitrite concentration which is the main metabolite of NO²⁹. This study seems to be in agreement with *Eberhardt et al.* who reported that the inhibitory effect of PPAR- α agonists on some inflammatory process are indirect and primarily due to superinduction of iNOS with high levels of NO³⁰. In addition to what mentioned before, *Tian et al.* had demonstrated that the endothelial protective effect of PPAR- δ agonists in diabetic mice is through eNOS signaling³¹. Finally, all the previous data suggesting that it has a benefit Gemfibrozil (antidyslipidemic, PPAR- α agonist) reduced jejunal and urinary bladder contractions, and has a relaxant effect on tracheal basal tone. This suggested that Gemfibrozil has a beneficial effect in obstructive airway diseases and cases of urgency and frequency of micturation and urinary incontinence. However, it may be used cautiously in cases of heart failure, GIT disturbances as constipation and prostatic hypertrophy.

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Table (1): Mean % reduction caused by gemfibrozil (25-800µg/ml) on the amplitude of contractions (cm) of isolated rabbit jejunum (Mean %±SEM).

Parameters	% Red	% Red	% Red	% Red	% Red	% Red	% Red	% Red
Dose								
Mean ± SEM	3.63±1.50	13.91±1.97	38.62±4.17	62.53±2.10	81.97±2.52	1.60±0.51	0.00±0.00	100.00±0.00
P	>0.05	*<0.001	*<0.001	*<0.001	*<0.001			–

% Red: percent reduction. ±SEM: standard error of mean *: significant (p < 0.05)

Table (2): Effect of gemfibrozil (40-1280µg/ml) on the basal tone (cm) of isolated tracheal spiral strips of guinea pig

Parameters	Gem 40µg/ml	Gem 80µg/ml	Gem 160µg/ml	Gem 320µg/ml	Gem 640µg/ml	Gem 1280µg/ml
Dose						
Mean ± SEM	0.03 ± 0.02	0.06 ± 0.02	0.10 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.16 ± 0.01
P	>0.05	*<0.05	*<0.001	*<0.001	*<0.001	*<0.001

Con: control Gem: gemfibrozil. ±SEM: standard error of mean. *: significant (p < 0.05).

Table (3): Mean % reduction caused by gemfibrozil (40-1280µg/ml) on acetylcholine (3µg/ml) induced contractions (cm) of isolated tracheal spiral strips of guinea pigs (Mean % ±SEM)

Parameters	% Red	% Red	% Red	% Red	% Red	% Red
Dose						
Mean ± SME	0.00±0.00	0.00±0.00	15.54±2.53	32.00±3.03	55.44±3.61	100.00±0.00
P	–	–	*<0.05	*<0.001	*<0.001	–

% Red: percent reduction. ±SEM: standard error of mean *: significant (p < 0.05)

Table (4): Mean % reduction caused by gemfibrozil (40- 1280µg/ml) on the amplitude of smooth muscles contractions (cm) of isolated urinary bladder strips of guinea pigs (Mean % ±SEM)

Parameters	% Red	% Red	% Red	% Red	% Red
Dose					
Mean ± SEM	7.29±2.44	18.29±2.03	31.31±1.80	76.93±1.99	98.54±1.04
P	*<0.05	*<0.001	*<0.001	*<0.001	*<0.001

% Red: percent reduction. ±SEM: standard error of mean *: significant (p < 0.05)

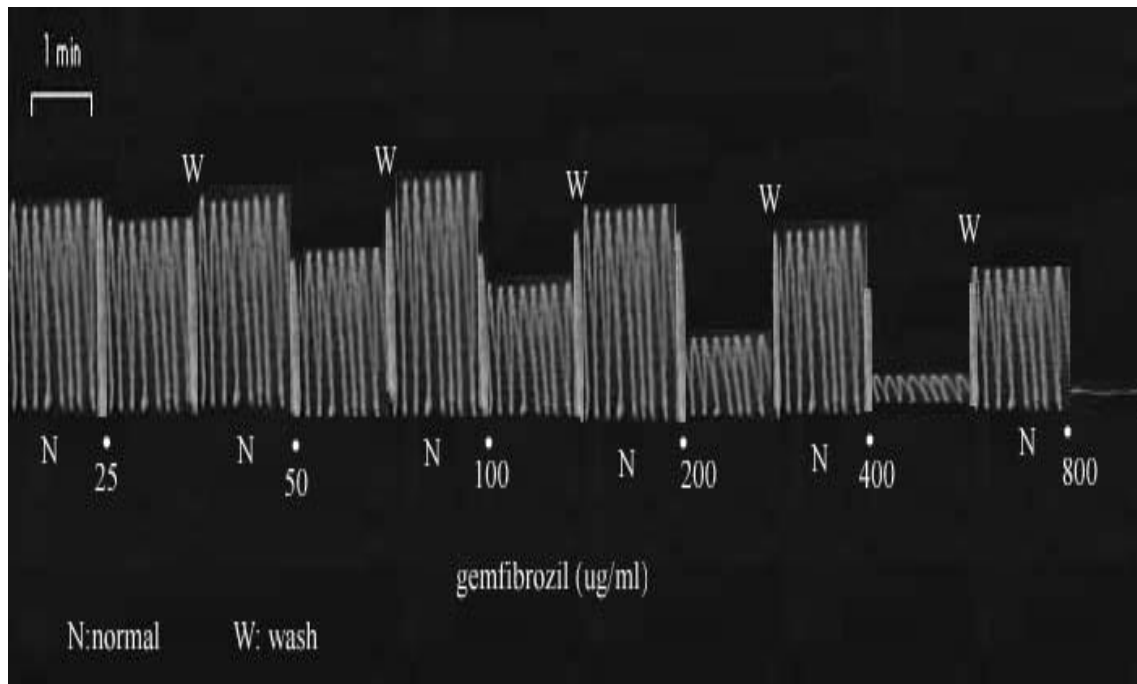


Fig. (1): Effect of gemfibrozil (25-800µg/ml) on the amplitude of contractions of isolated rabbit jejunum.

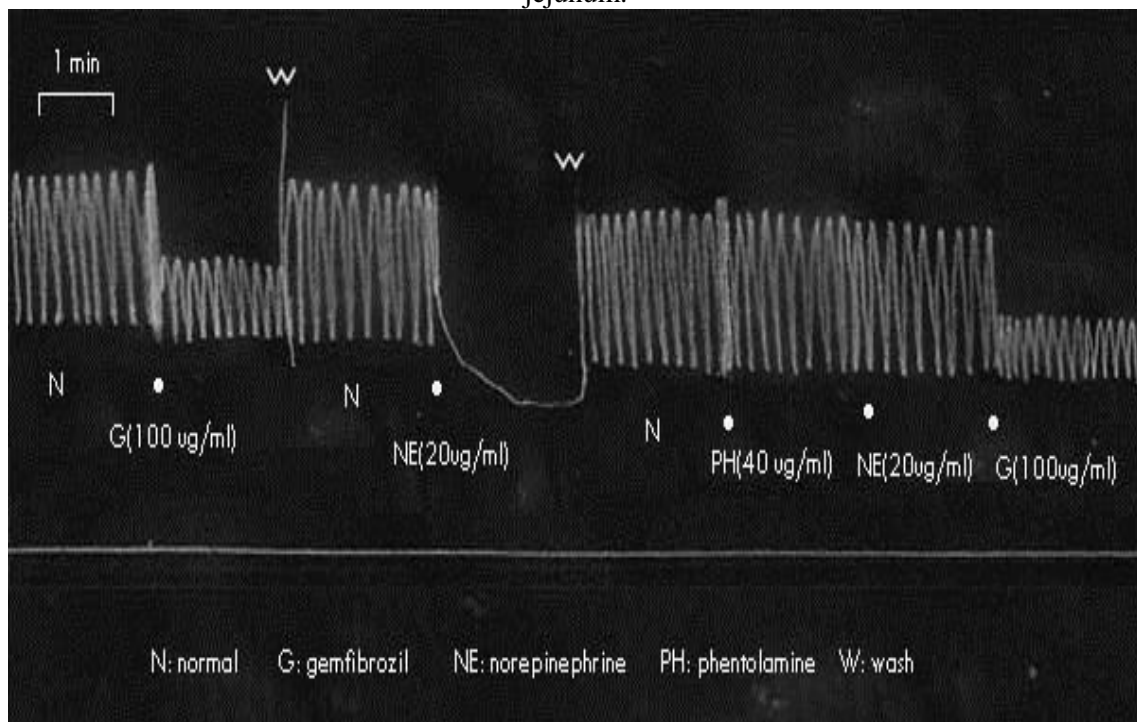


Fig. (2): Effect of gemfibrozil (100µg/ml) on the amplitude of contractions of isolated rabbit jejunum after complete blockade of alpha adrenergic receptors

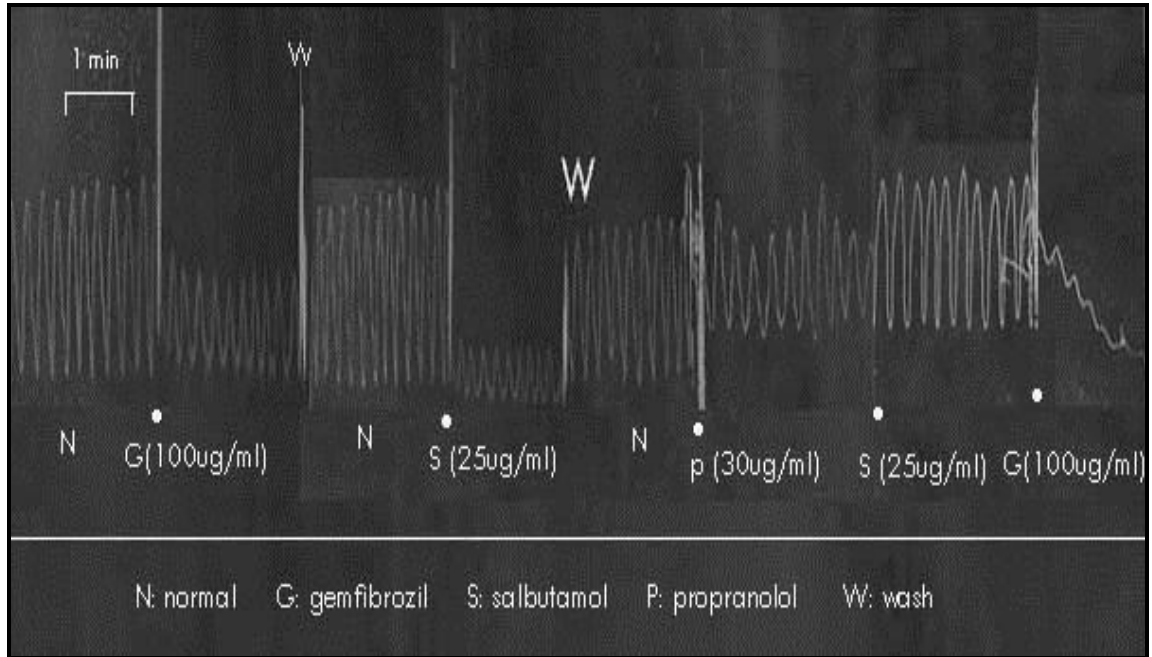


Fig. (3): Effect of gemfibrozil (100 μ g/ml) on the amplitude of contractions of isolated rabbit jejunum after complete blockade of beta adrenergic receptors.

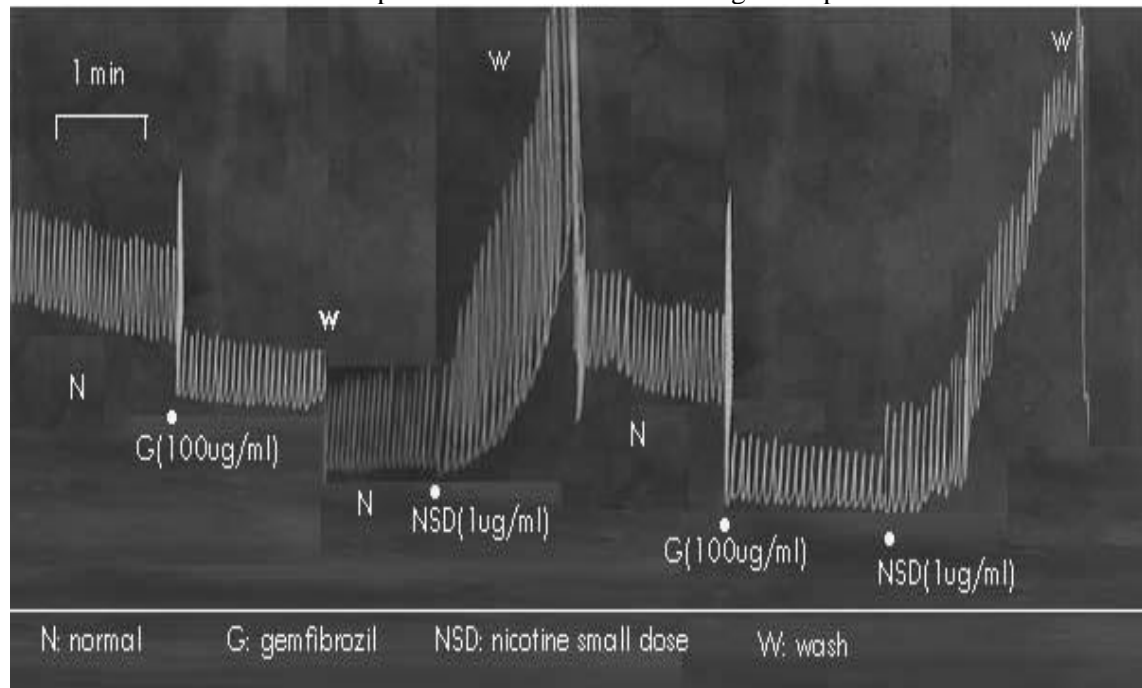


Fig. (4): Effect of gemfibrozil (100 μ g/ml) on nicotinic receptors in isolated rabbit jejunum.

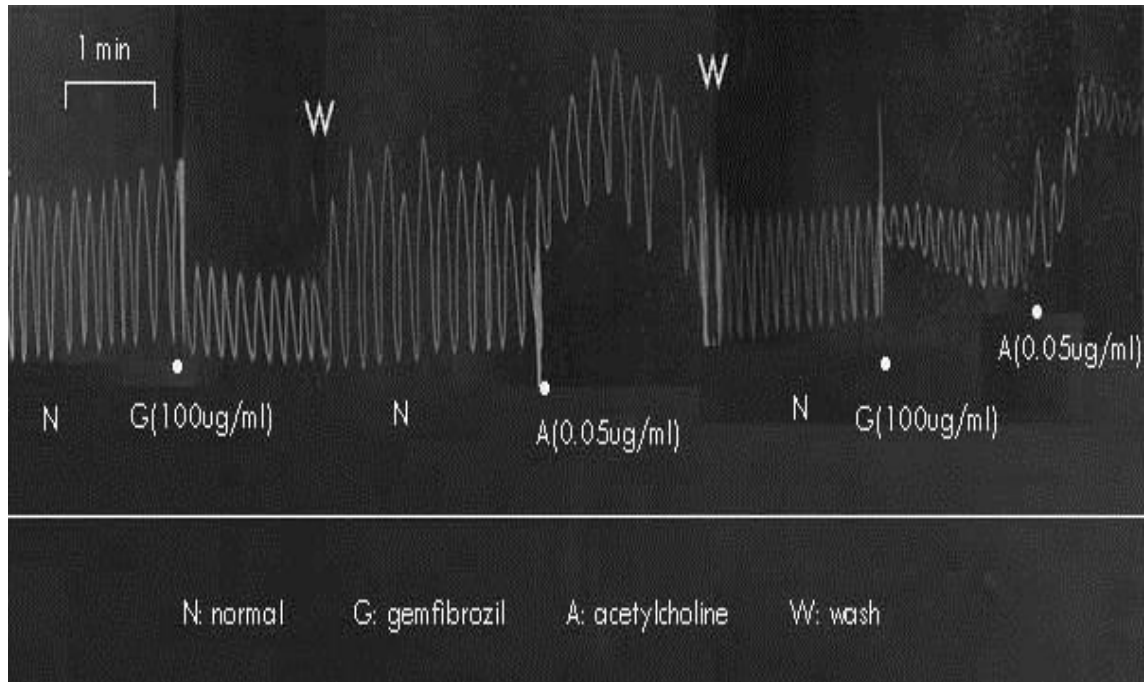


Fig. (5): Effect of gemfibrozil (100µg/ml) on muscarinic receptors in isolated rabbit jejunum.

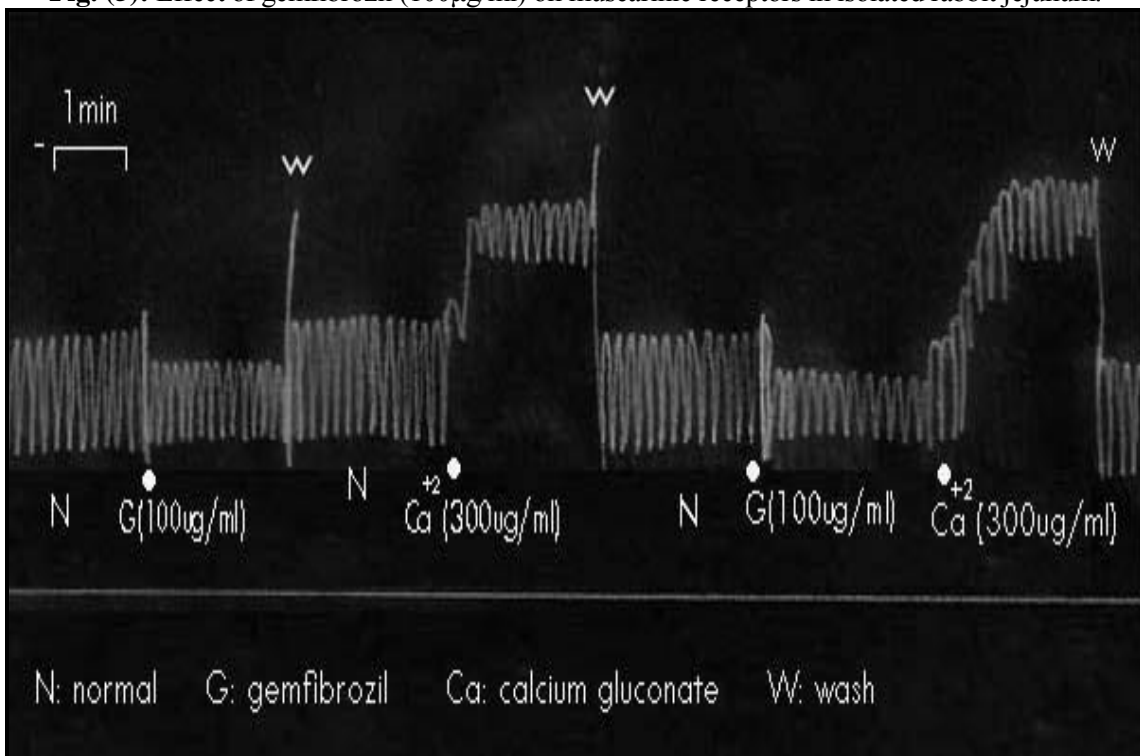


Fig. (6): Effect of gemfibrozil (100µg/ml) on calcium channels in isolated rabbit jejunum.

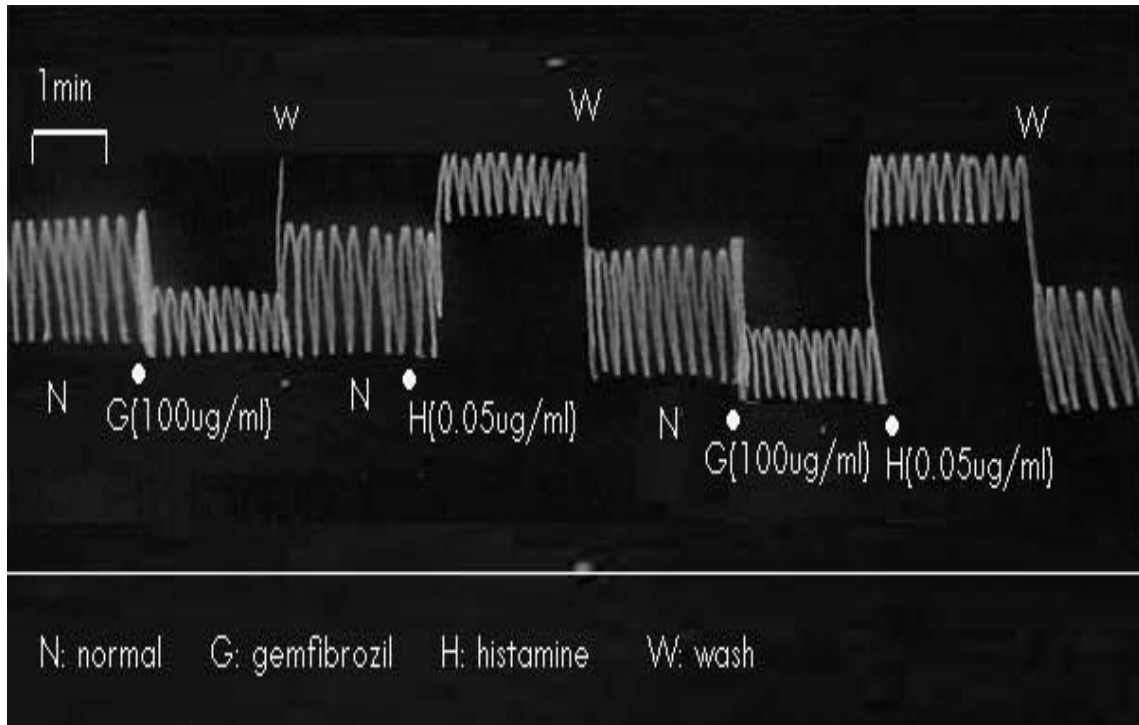


Fig. (7): Effect of gemfibrozil (100 μ g/ml) on histaminic receptors in isolated rabbit jejunum.

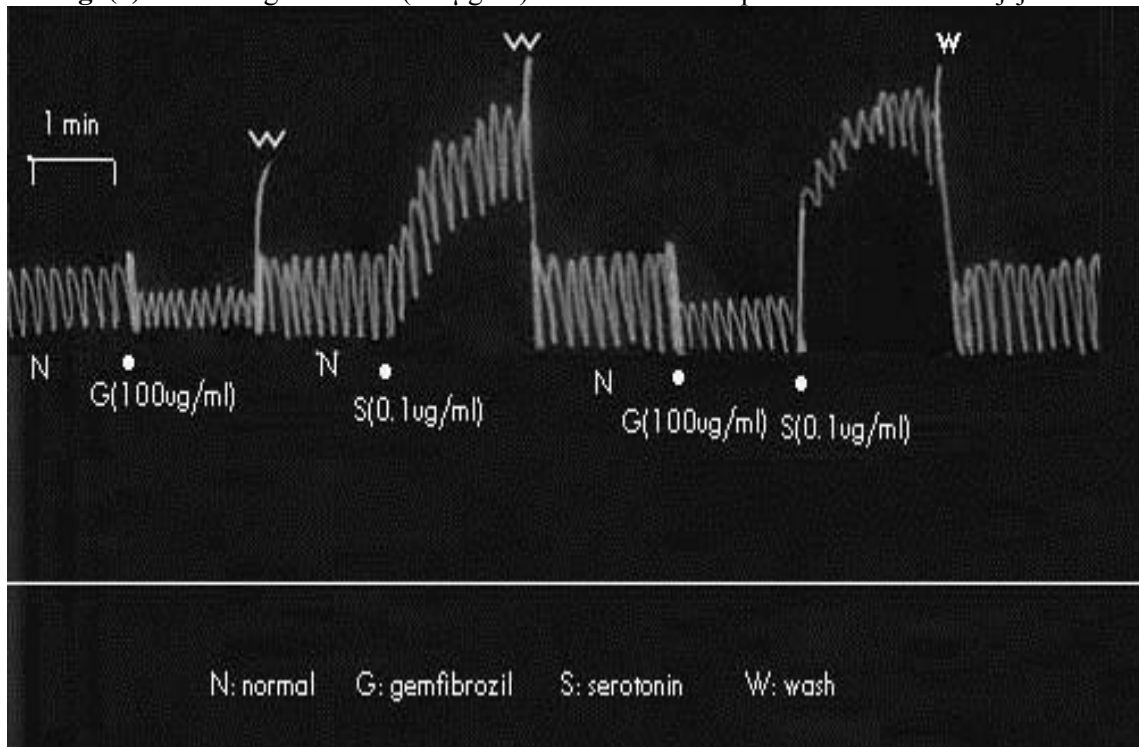


Fig. (8): Effect of gemfibrozil (100 μ g/ml) on serotonin receptors in isolated rabbit jejunum.

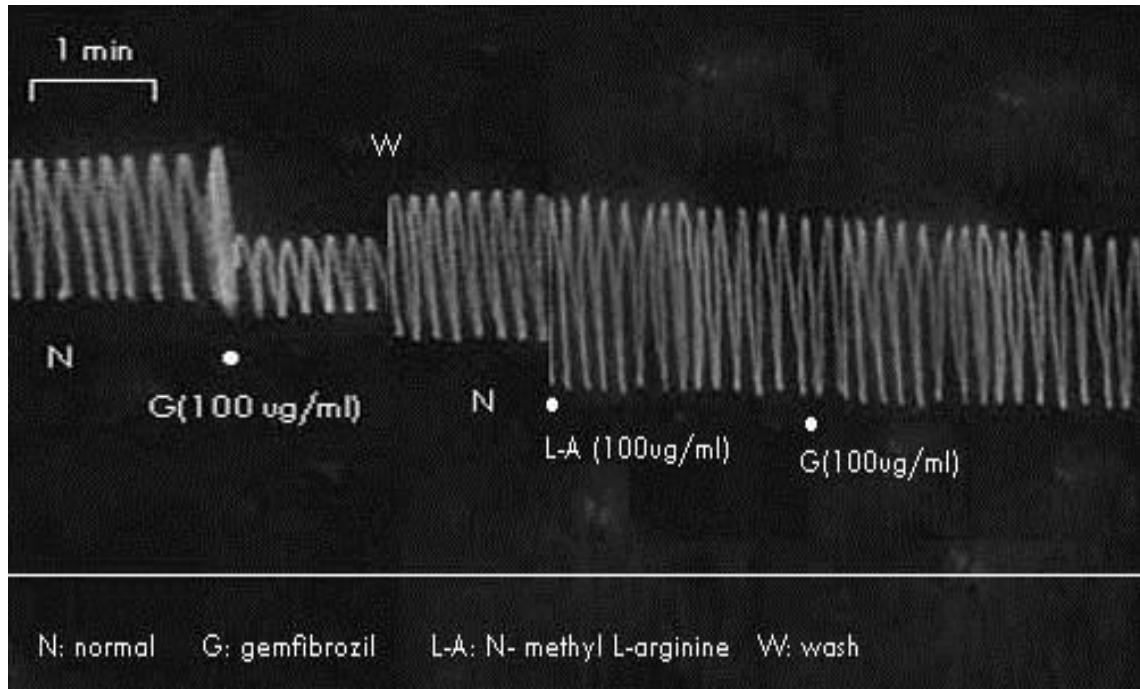


Fig. (9): Effect of gemfibrozil (100µg/ml) on the amplitude of contractions of isolated rabbit jejunum after inhibition of nitric oxide synthase by N-methyl L- arginine (100µg/ml).

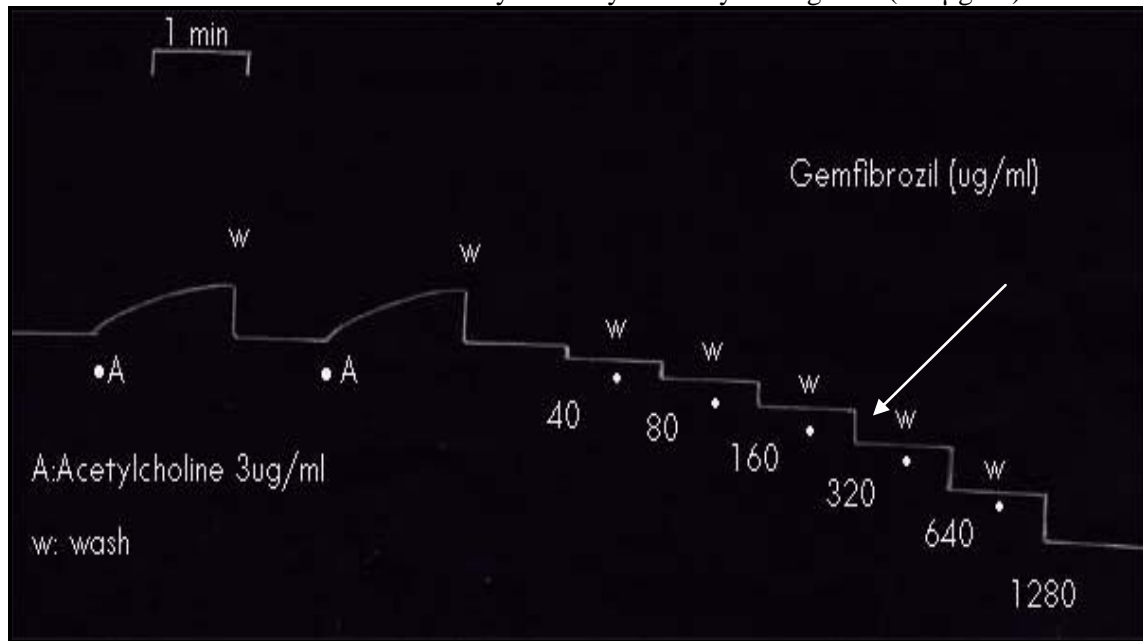


Fig. (10): Effect of gemfibrozil (40-1280µg/ml) on the basal tone of isolated tracheal spiral strips of guinea pigs.

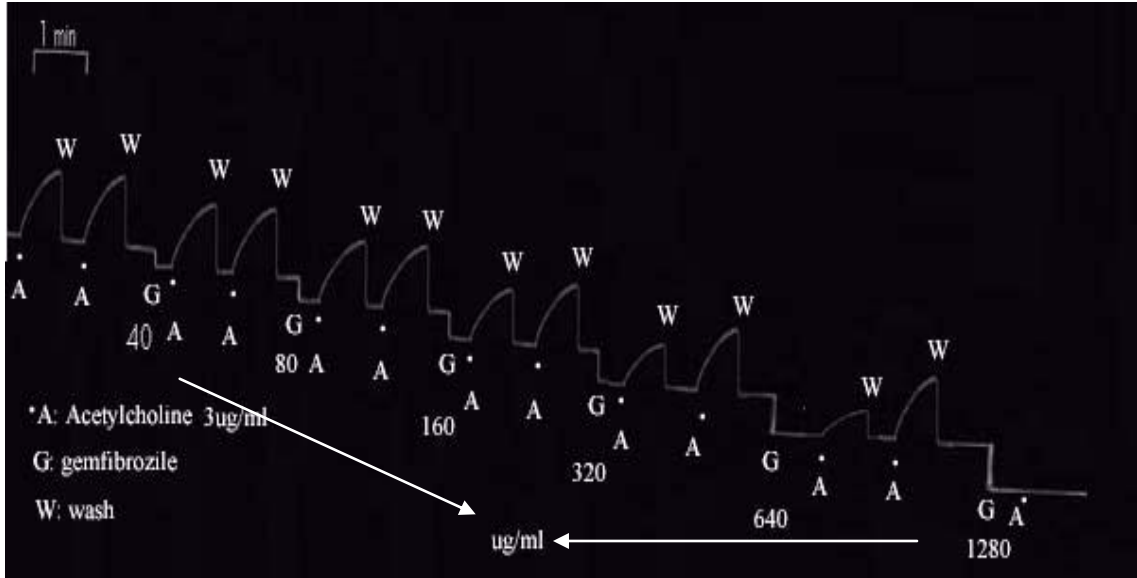


Fig. (11): Effect of gemfibrozil (40-1280µg/ml) on acetylcholine (3µg/ml) induced contractions of isolated tracheal spiral strips of guinea pigs.

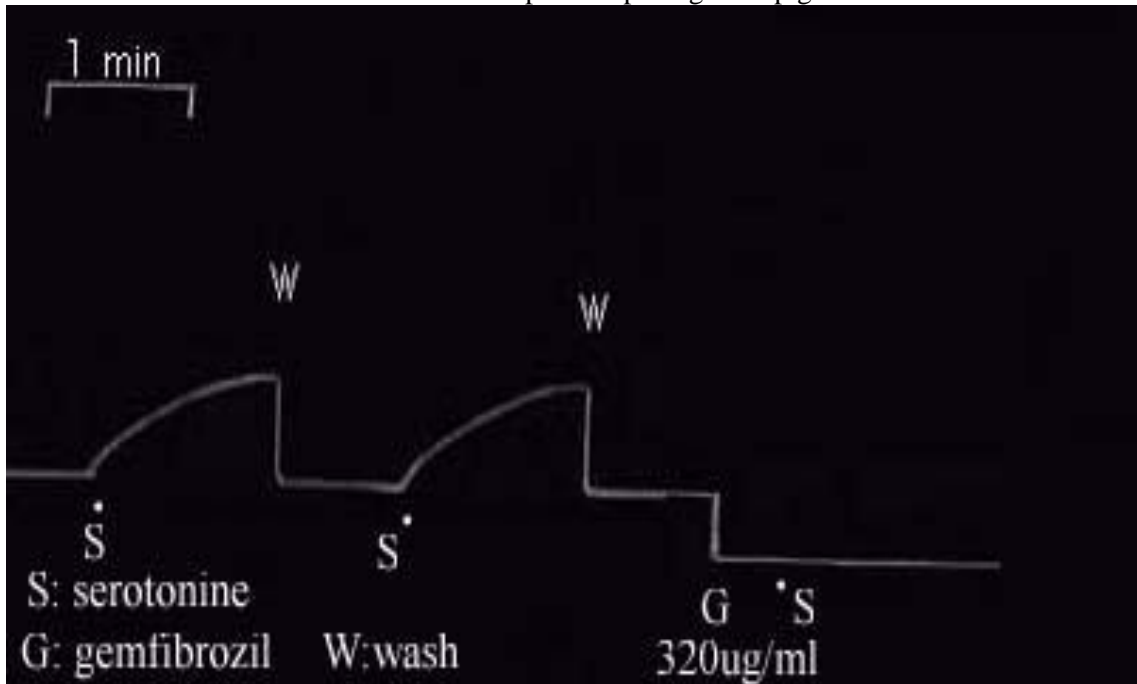


Fig. (12): Effect of gemfibrozil (320µg/ml) on the amplitude of serotonin (5µg/ml) induced contractions of isolated tracheal spiral strips of guinea pigs.

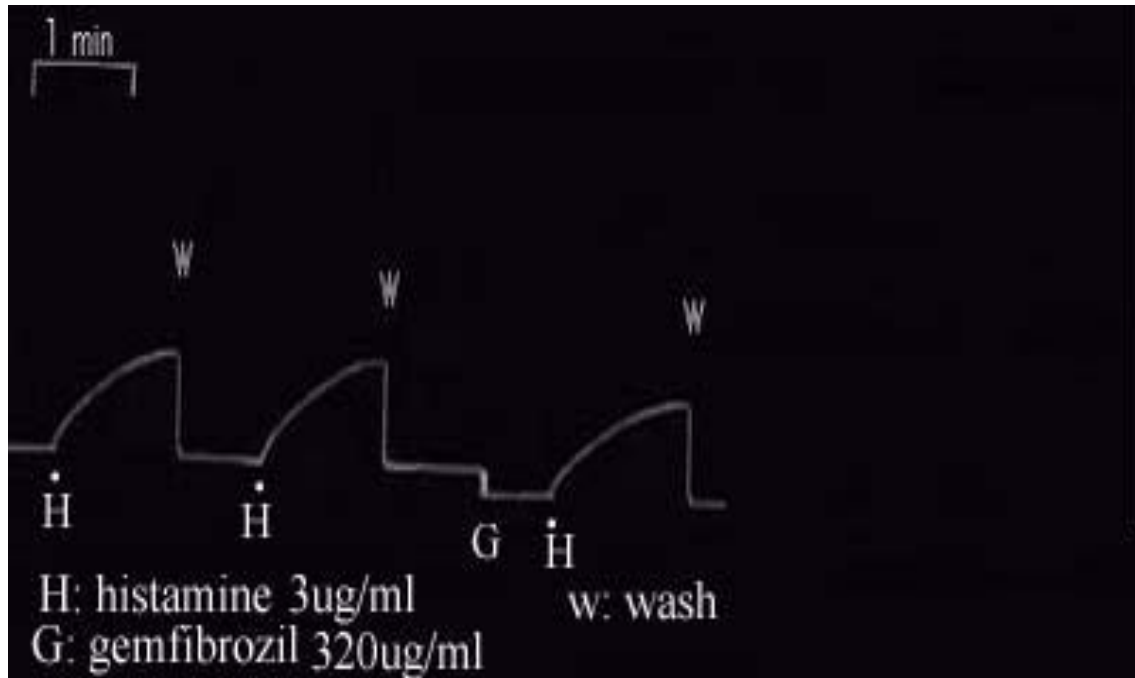


Fig. (13): Effect of gemfibrozil (320µg/ml) on the amplitude of histamine (3µg/ml) induced contractions of isolated tracheal spiral strips of guinea pigs.

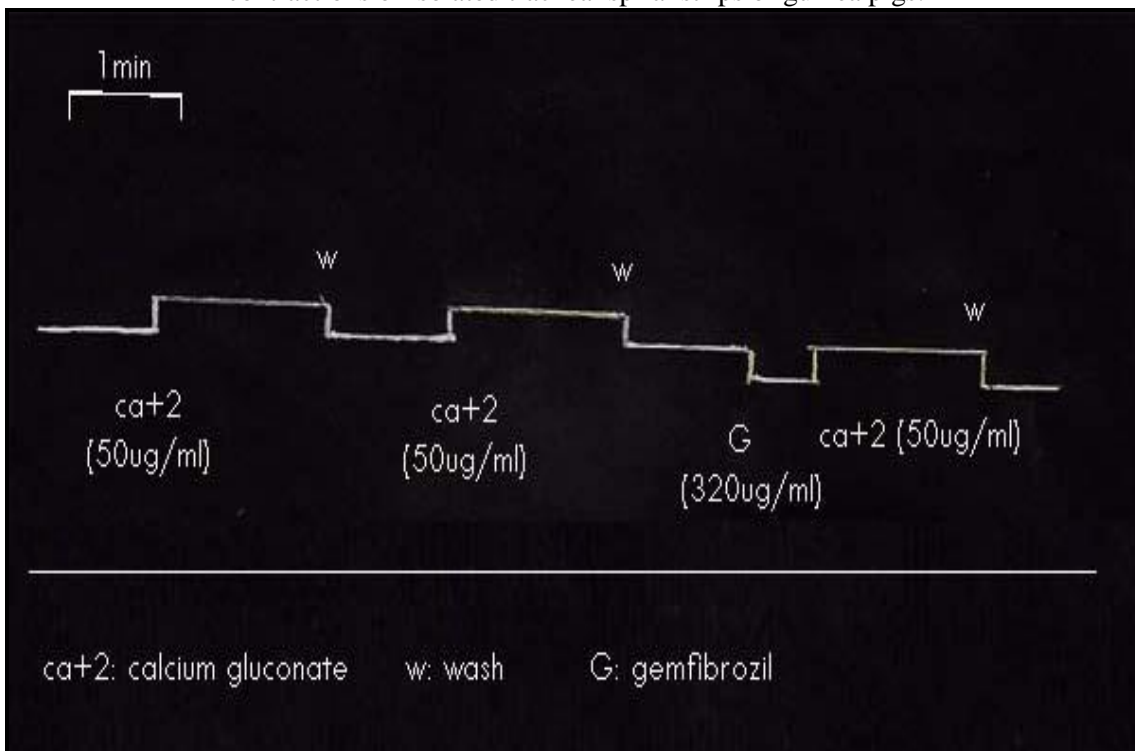


Fig. (14): Effect of gemfibrozil (320µg/ml) on the amplitude of calcium (50µg/ml) induced contractions (cm) of isolated tracheal spiral strips of guinea pigs.

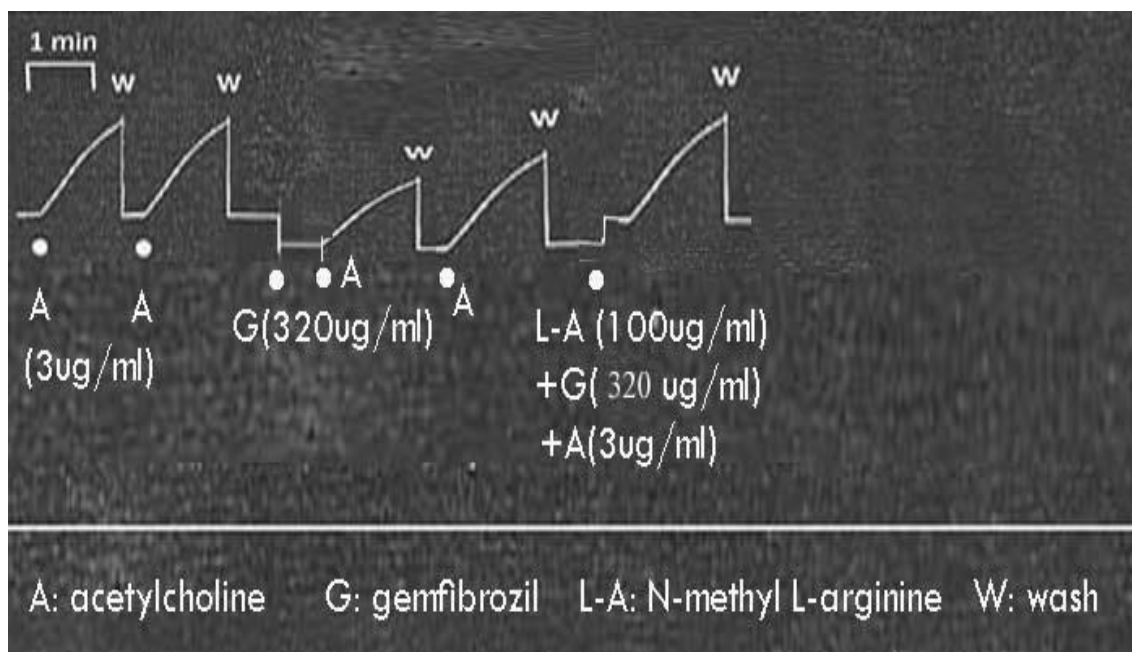


Fig. (15): Effect of gemfibrozil (320µg/ml) on the amplitude of acetylcholine (3µg/ml) induced contractions (cm) of isolated tracheal spiral strips of guinea pigs after inhibition of NO synthase by N-methyl L-arginine (100µg/ml).

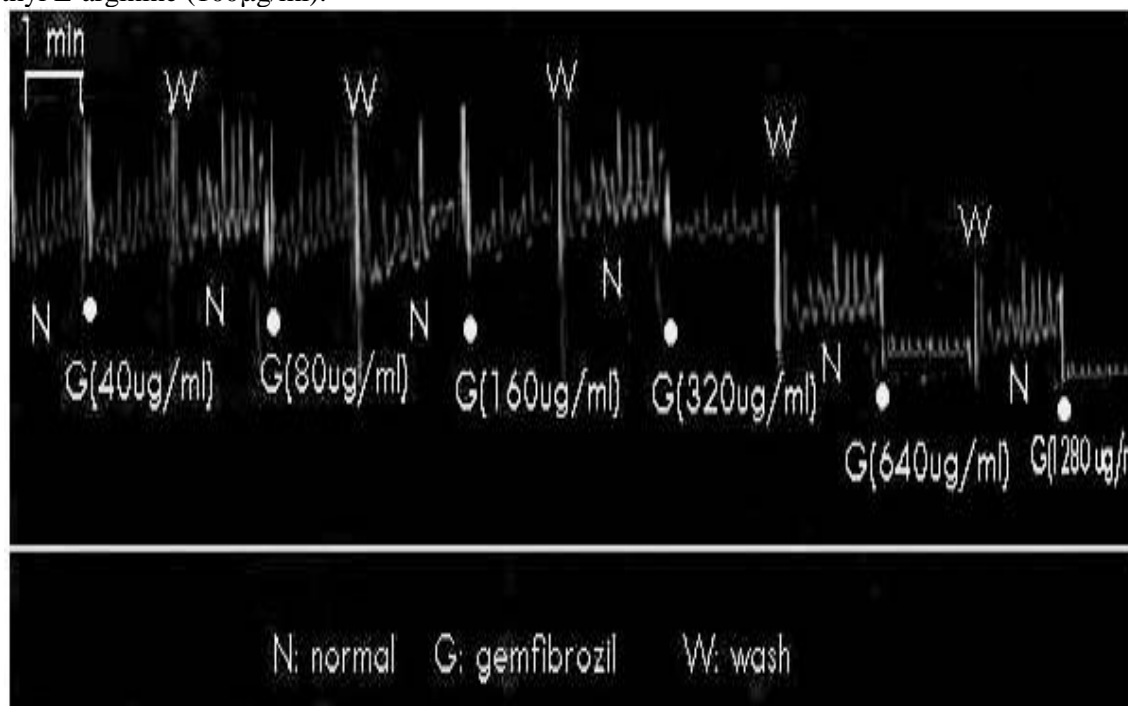


Fig. (16): Effect of gemfibrozil (40-1280 mcg/ml) on the amplitude of smooth muscles contractions of isolated urinary bladder strips of guinea pigs.

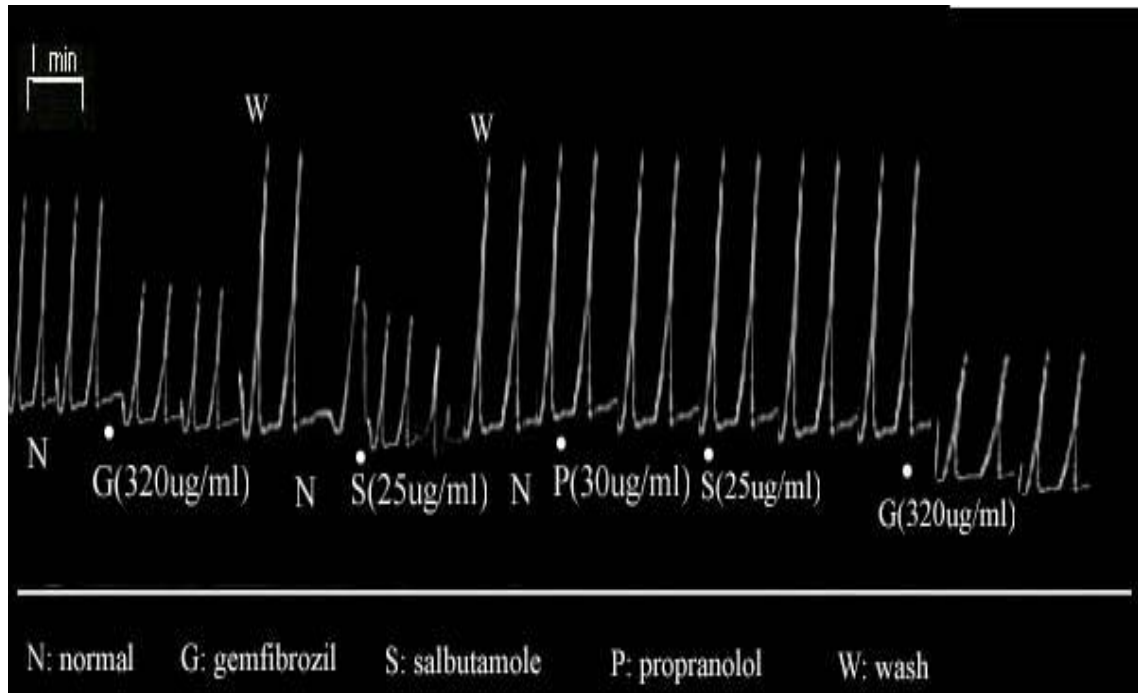


Fig. (17): Effect of gemfibrozil (320µg/ml) on the amplitude of smooth muscle contractions of isolated urinary bladder strips of guinea pigs after complete blockade of beta adrenergic receptors.

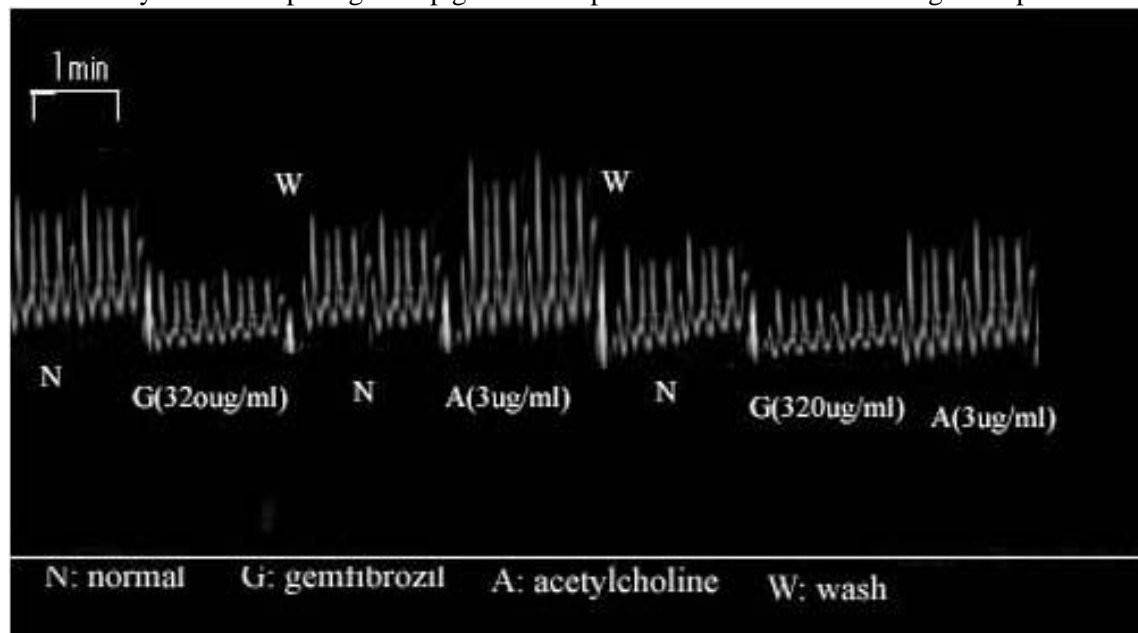


Fig. (18): Effect of gemfibrozil (320µg/ml) on muscarinic receptors in isolated urinary bladder strips of guinea pigs.

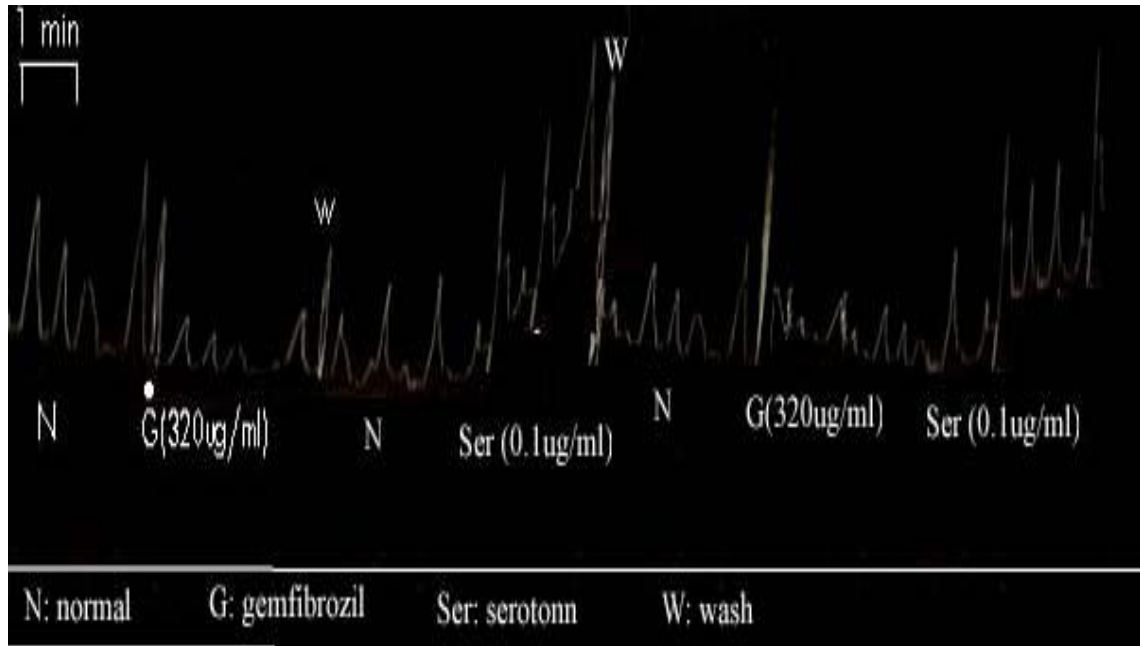


Fig. (19): Effect of gemfibrozil (320µg/ml) on serotonin receptors in isolated urinary bladder strips of guinea pigs.

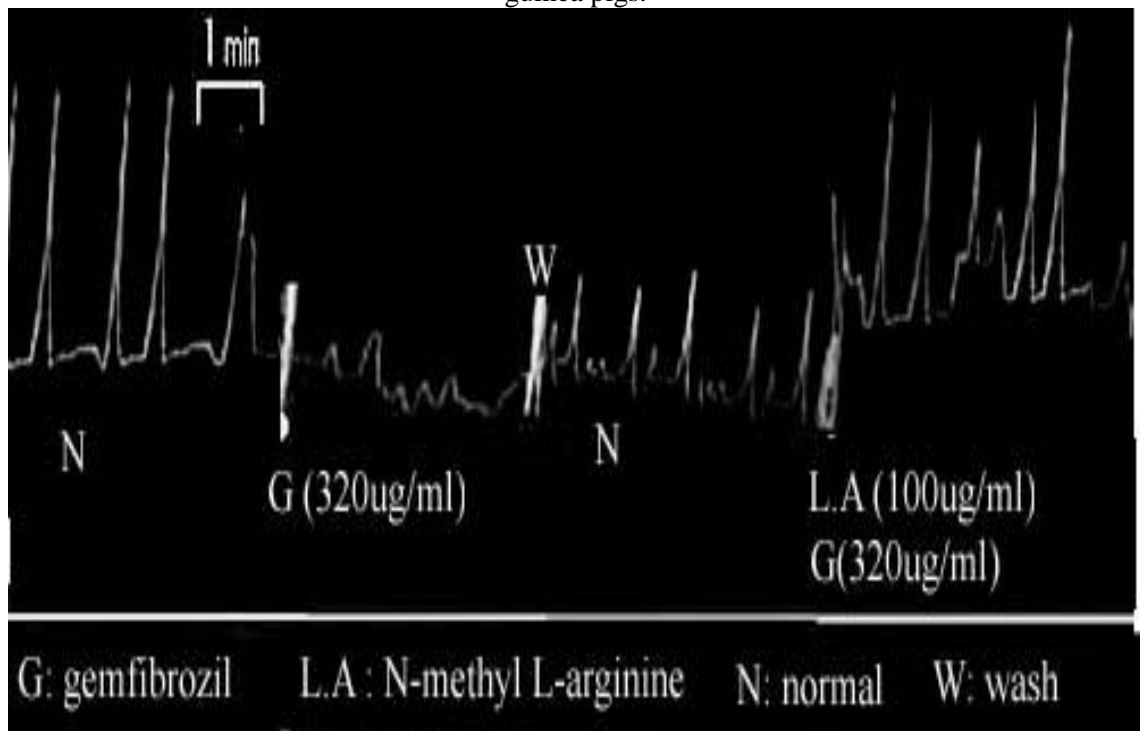


Fig. (20): Effect of gemfibrozil (320µg/ml) on the amplitude of smooth muscle contractions of isolated urinary bladder strips of guinea pigs after inhibition of NO synthase by N-methyl L-arginine (100µg/ml).